THE PIGMENTS OF *MICROSPORUM COOKEI**

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Microsporum cookei, dermatophyte species recently described by Ajello (1), produces macroconidia which resemble those of *M. gypseum* in shape, but which have tremendously thickened cell walls. When grown in culture on Sabouraud's agar, *M. cookei* produces a purplish-red pigment which diffuses into the medium (1). The present study is concerned with the chemical and physical properties of this pigment.

A number of other species of dermatophytes, including *Trichophyton rubrum*, *T. violaceum*, and *M. canis*, are known to produce pigments, and the pigment of *T. rubrum* especially has been studied by a number of investigators. Sabouraud (2) reported that the pigments of dermatophytes were acids, and could be precipitated by alkali. Tate (3), in a brief account devoid of experimental detail, found that the red to reddish-brown pigments of several species, including *T. rubrum*, *T. mentagrophytes*, and *T. megnini*, are soluble in dilute acids and acid alcohol, but are only very slightly soluble in alkali. This pigment has the property of a pH indicator, changing reversibly from yellow in acid solution to red or reddish-brown in alkali. The pigment also has the property of an oxidation-reduction indicator, since alkaline solutions are reduced by sodium hydrosulfite, and can be reoxidized by atmospheric oxygen. Tate concluded that the pigment resembles the yellow pigment of the lichen *Physcia*, and appears to be an anthracene derivative. The yellow pigment of *Physcia*, known as physcion, is 1,8-dihydroxy-3-methyl-6-methoxyl anthraquinone (4).

Tate's findings were confirmed by Thompson (5), who found that the pigments of *T. rubrum* and *T. mentagrophytes* are soluble in acetone. The red color of aqueous acetone extracts becomes blue-lavender above pH 9. Upon standing in alkaline solution, the lavender color changes to yellow, but returns to lavender upon oxygenation.

Lewis and Hopper (6) investigated some of the cultural conditions necessary for pigment production by *T. rubrum* and *M. canis*. Both species produce their characteristic pigments on media containing dextrose, levulose, mannose, crude maltose, mannitol, honey, or corn syrup, but not on media in which galactose, lactose, sucrose, pure maltose, raffinose, inulin, or starch is the carbon source. Addition of vitamins to the media, or changes in its pH within the range 4.0–7.0, had no appreciable effect upon pigment production. The pigments of both *T. rubrum* and *M. canis* were fluorescent when examined under ultraviolet light. The findings of Lewis and Hopper concerning the dependence of pigment production upon the nature of the carbon source were substantiated by the work of Bocobo and Benham (7) with 40 strains of *T. rubrum*, 38 of which produced a purple to red pigment on corn meal dextrose agar. None produced pigment in the presence of dextrose, however, when ammonium chloride was the nitrogen source, indicating that this factor as well as the nature of the carbohydrate may influence pigment production. On various media of pH 4.0–4.5, *T. rubrum* produced a yellow pigment.

Georg and Maechling (8) examined a variant of *T. mentagrophytes* characterized by greatly increased production of an orange-red pigment which was completely confined to the cells of the mycelium, and was extractable with water and alcohol, but not by chloroform or other organic solvents except following treatment with glacial acetic or mineral acids. Since this pigment was reduced by sodium hydrosulfite with a color change, and was reoxidized by contact with air, these workers concluded that it was an anthraquinone derivative.

According to Pinetti (9), the cultural conditions for pigment production by *T. violaceum* include glucose or glycerine as a carbon source, glycine as a nitrogen source, a pH of 5.9–6.8, and a temperature optimum of 28°C. The pigment was insoluble in water or organic solvents, but could be extracted by aqueous ammonia at pH 12. Upon readjustment to pH 8, some degree of separation of the mixture of pigments present was achieved by chromatography on an alumina column (10).

Johnson and Reedal (11) determined a number of properties of the pigment of *M. gypseum*, which is insoluble in organic solvents, but extractable with NaOH. While the pigment is an indicator, it is not reduced by sodium hydrosulfite.

The nutritional factors conditioning pigment production by *T. mentagrophytes*, *T. rubrum*, *T. megnini*, and *T. gallinae* were studied by Silva (12), who found that vitamins were not required, and confirmed the finding of Bocobo and Benham (7) concerning the necessity of an organic nitrogen source. Experiments with single amino acids indicated that the four species had differing amino acid requirements for pigment formation.

More recent studies of the pigment of *T. rubrum* have been made by Wirth *et al.* (13) and Mier (14). Wirth *et al.* (13) observed that the possible identification by earlier workers of dermatophyte pigments as anthraquinones "is completely un-

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supported by published experimental evidence". Following growth of *T. rubrum* on a liquid medium, the mycelium was extracted by these workers with ethylene dichloride and the fats were removed with cyclohexane leaving a residue of "crude red pigment". The pigment mixture was chromatographed on columns of Terra Alba No. 1 plus celite, using ethylene dichloride containing 0.1 per cent glacial acetic acid as the developing solvent, and proved to be very complex. From this mixture red needles, orange plates, and purple needles were isolated in crystalline form. Nitrogen was not present in either the red needles or orange plates. Because of differences in the solubilities of these three compounds in $NaHCO_3$ or Na_2CO_3, and the differences in the colors developed in acids, base, and after reduction with sodium dithionite, it was clearly demonstrated that the coloring matter of *T. rubrum* is not a single substance, but a mixture of several different compounds.

Mier (14) extracted the pigment of *T. rubrum* with glacial acetic acid, partitioned into chloroform, and by paper chromatography, using chloroform:petroleum ether 7:3 as the developing solvent, separated three major fractions, one yellow, one orange-red, and one deep bluish-red. Two of these three fractions were isolated in crystalline form. On the basis of their absorption spectra, fluorescence, color changes with alkali, and reversible reduction with sodium hydrosulfite and oxidation by atmospheric oxygen, Mier concluded that these pigments were polyhydroxy-2-methyl anthraquinones.

The studies of Mier (14) with *T. rubrum* were subsequently extended by McCabe and Mier (15) to include *T. mentagrophytes* and *T. violaceum* as well. Chloroform extracts of the pigments of *T. mentagrophytes*, obtained by the technic of Mier (14) were separable into two fractions upon the addition of methanol, and those of *T. violaceum* could be separated into two fractions by the addition of petroleum ether. By subsequent paper chromatography and ultraviolet spectrophotometry of the fractions eluted from paper chromatograms, the existence of at least five pigments was demonstrated, which these workers designated A_1, A_2, B, C_1, and C_2. The colors of these pigments in chloroform solution were deep yellow, bright yellow, orange-red, purple, and yellow respectively. *T. violaceum* was found to contain all five of these pigments, while *T. rubrum* contained A_2, B, C_1, and C_2, and *T. mentagrophytes* contained only A_1 and B.

Baichwal and Walker (16) extracted the pigment of *T. rubrum* with 5% sodium hydroxide, acidified and extracted this solution with chloroform, and then extracted the chloroform solution with 5% sodium bicarbonate. Evaporation *in vacuo* gave a light yellow amorphous powder, whose absorption spectrum was very similar to that of the material extracted by ethylene dichloride. Using an acid alumina column with ethylene dichloride plus 1% glacial acetic acid as the developing solvent, four bands were resolved, yellowish, light brown violet, diffuse brown, and brown. Absorption spectra in methanol were taken in both the visible and ultraviolet regions.

According to Zussman *et al.* (17) the environmental conditions favoring pigmentation in *T. rubrum* include the presence of glucose as a carbon source, tyrosine or phenylalanine as a nitrogen source, and oxygen. Vitamins are not required. Using extraction and purification methods similar to those of Wirth *et al.* (13), Zussman *et al.* (18) obtained from *T. rubrum* a yellow pigment, a red one, an orange one, and a purple one. Studies of the solubilities of these components in various solvents and their decomposition temperatures suggested that the purple pigment differed appreciably from the other three. Since the purple pigment and the yellow pigment were found to contain nitrogen, and since the yellow pigment was found to have a high specific activity following growth in the presence of randomly labeled tyrosine or tyrosine labeled in the 2-position with C^{14}, it was concluded that the pigments of *T. rubrum* were melanoid in nature.

MATERIALS AND METHODS

The culture studied, *Microsporum cookei* A-999, was obtained from Dr. Libero Ajello, Communicable Disease Center, Mycology Unit, Chamblee, Ga. *Trichophyton rubrum* 2680 was obtained from Dr. Norman F. Conant, Duke University School of Medicine, Durham, N. C., and was studied for purposes of comparison. Stock cultures were maintained on slants of Sabouraud's dextrose agar, and were transferred weekly to avoid pleomorphism. Inoculations were made on plates of Sabouraud's dextrose agar at numerous points, and the plates were incubated at room temperature for three weeks.

Twelve plates in which suitable development of pigment occurred were first flooded with a dilute solution of lysol, and then rinsed in several changes of distilled water. Extraction of the pigment and paper chromatography were performed following the technic of Mier (14). The agar cultures were first extracted in glacial acetic acid by grinding in a mortar with sand. The acetic acid extract was filtered, and 150 ml. of filtrate was diluted to 1000 ml. with distilled water. The pigments were then extracted into 300 ml. of chloroform in a separatory funnel. The chloroform solution was washed repeatedly with water to remove all traces of acid, and the water was removed by anhydrous sodium sulphate. The chloroform extract was then concentrated by evaporation under reduced pressure to a volume of 5 ml.

Strips of Whatman 3MM paper were spotted with the chloroform extract, and ascending paper chromatography was performed using a developing solvent of 70% chloroform and 30% petroleum ether (v/v).

After numerous trials, it was found that sucrose was a suitable adsorbent, and sucrose columns measuring 35 × 1.5 cm. were used for fractiona-

tion of the chloroform extract. The developing solvent was the same as that used in paper chromatography.

Absorption spectra of the pigment fractions in the visible and ultraviolet regions were determined in a number of solvents using a Beckman model DU spectrophotometer. Spots were cut from the paper chromatograms and eluted with the proper solvent. To determine indicator properties of the pigments, spots cut from paper chromatograms were eluted with phosphate buffers of different pH values. In studying the oxidation-reduction phenomena exhibited by the pigments in alkaline solution, 0.1 ml. of a 50% aqueous solution of sodium hydrosulfite was added to 1.0 ml. of the pigment solution in N/10 base. Reoxidation was achieved by bubbling air through the solutions.

To detect the presence of polyhydroxy anthraquinones, paper chromatograms were sprayed with a 0.5% solution of magnesium acetate in methanol, and were dried in an oven at 90°C. for 5 minutes (19).

The details of methods used in obtaining the pigment fractions in solid form will be included in the results. A Fisher-Johns melting point apparatus was used in the study of thermal decomposition of the pigments. The Hinsberg test for the presence of nitrogen was done according to the procedure of Fieser (20) using benzenesulfonyl chloride.

The fluorescence spectrum was obtained using a model DU Beckman spectrophotometer modified by having the sample tube in the position of the usual light source. Excitation was provided by a General Electric 100 watt mercury lamp, type S-4. The photomultiplier was operated at full sensitivity, with a slit width of 2 mm.

In obtaining infrared absorption spectra, the pigment fractions in KBr pellets were examined using a model 21 Perkin Elmer spectrophotometer.

RESULTS

The chloroform extracts obtained from *M. cookei* were spotted onto strips of Whatman No. 3 MM paper, and chromatographed using chloroform:petroleum ether 7:3 as the developing solvent. Four spots were consistently found on the resulting chromatograms: a pale yellow spot, R_f 0.19; a purple spot, R_f 0.41; an orange-brown spot, R_f 0.67; and a bright yellow spot, R_f 0.74. Chloroform extracts of *T. rubrum* gave chromatograms of a similar appearance, and with closely similar R_f values. Cochromatograms of the combined extracts of the two fungi indicated the identity of the pigments of *M. cookei* with those of *T. rubrum*.

The pale yellow pigment was present only in trace amounts, and efforts to obtain quantities sufficient for detailed study were unsuccessful. When the orange-brown pigment was eluted with chloroform and rechromatographed, paper strips with the same appearance as the original chromatograms of the whole chloroform extract

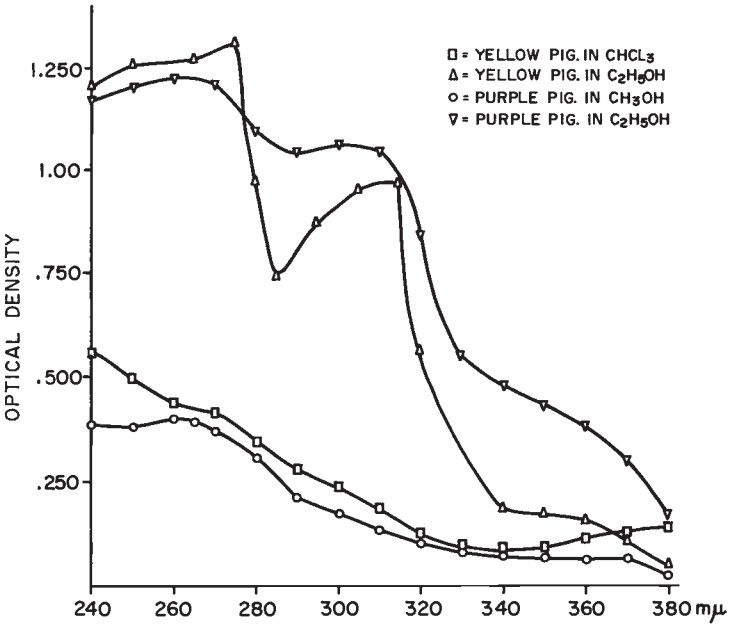

FIG. 1. Absorption spectrum of the pigments of *M. cookei* in organic solvents in the ultraviolet region

of *M. cookei* were obtained. It was concluded, therefore, that this spot represented a mixture of pigments. The studies to be reported were consequently restricted to the bright yellow and purple pigments, which appeared to be homogeneous on rechromatography.

Upon spraying the chromatograms with 0.5 % magnesium acetate in methanol and then subjecting the papers to a temperature of 90°C. for five minutes (19), the bright yellow spot turned pink and the color of the purple spot was intensified. This test is supposedly specific for anthraquinone derivatives having at least one hydroxyl group in an alpha-position.

The bright yellow pigment could be eluted from the chromatograms with chloroform. The purple pigment was not eluted with this solvent, but was eluted with methanol. Absorption spectra of the yellow pigment in chloroform, of the purple pigment in methanol, and of both pigments in ethanol, N/10 HCl, N/10 NaOH, and pH 7.0 phosphate buffer are presented in

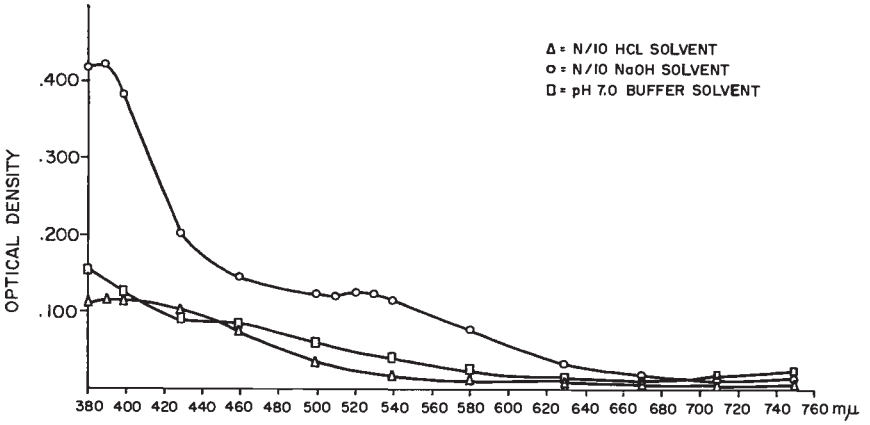

FIG. 2. Absorption spectra of the pigments of *M. cookei* in organic solvents in the visible region

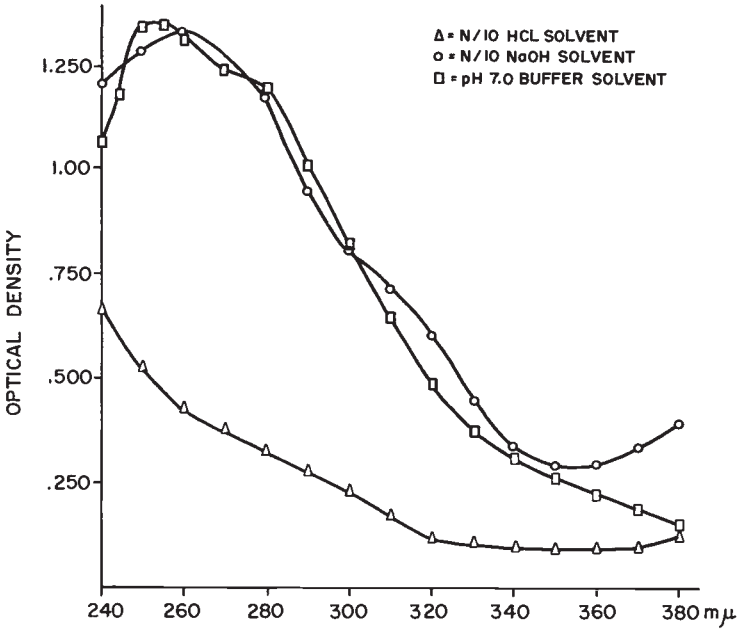

FIG. 3. Absorption spectra of the bright yellow pigment of *M. cookei* in N/10 HCl, N/10 NaOH and pH 7.0 buffer in the ultraviolet region

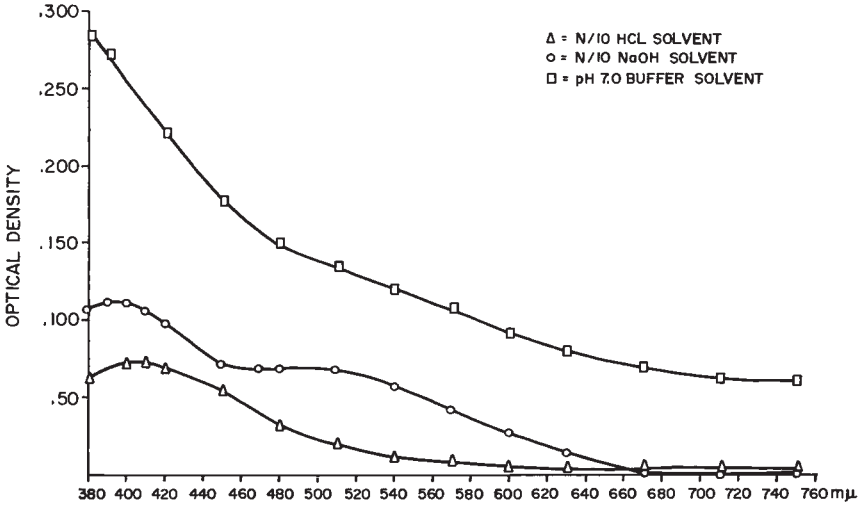

FIG. 4. Absorption spectra of the bright yellow pigment of *M. cookei* in N/10 HCl, N/10 NaOH and pH 7.0 buffer in the visible region

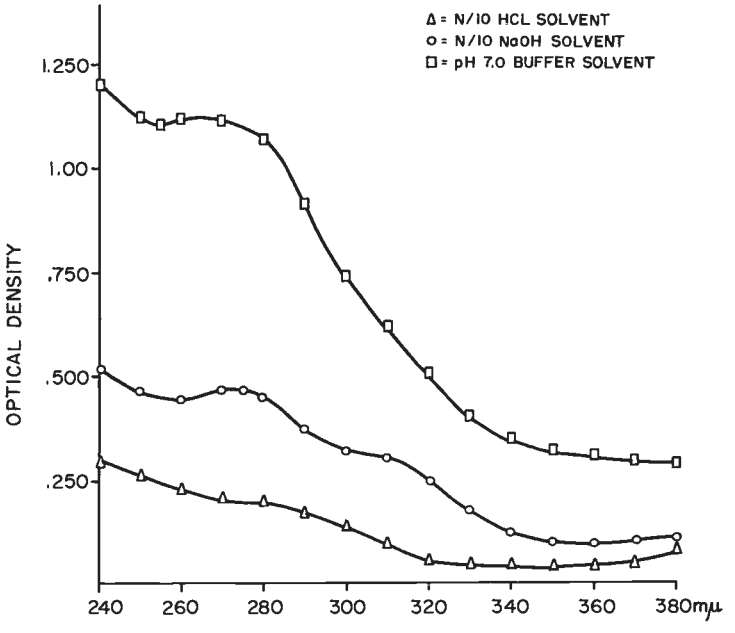

FIG. 5. Absorption spectra of the purple pigment of *M. cookei* in N/10 HCl, N/10 NaOH and pH 7.0 buffer in the ultraviolet region

Figures 1–6. The yellow pigment has a peak at 270–275 mμ in chloroform, at 270 mμ in base, and at 250–255 mμ in pH 7.0 buffer. This peak is not present in acid solution, but is replaced by two peaks, at 270 and at 310–315 mμ in ethanol. No peaks occur in the visible region in chloroform or in pH 7.0 buffer. In N/10 HCl, there is a peak at 390–400 mμ, in ethanol at 470 mμ, and in N/10 NaOH there are two peaks, at 390 mμ and at 520–530 mμ.

The purple pigment has a prominent absorption maximum in the ultraviolet, which occurs at 260–265 mμ in methanol, 270–280 mμ in N/10 HCl, 270–275 mμ in N/10 NaOH, and 250–275 mμ in pH 7.0 buffer. In ethanol, two peaks are present in the ultraviolet at 250–280

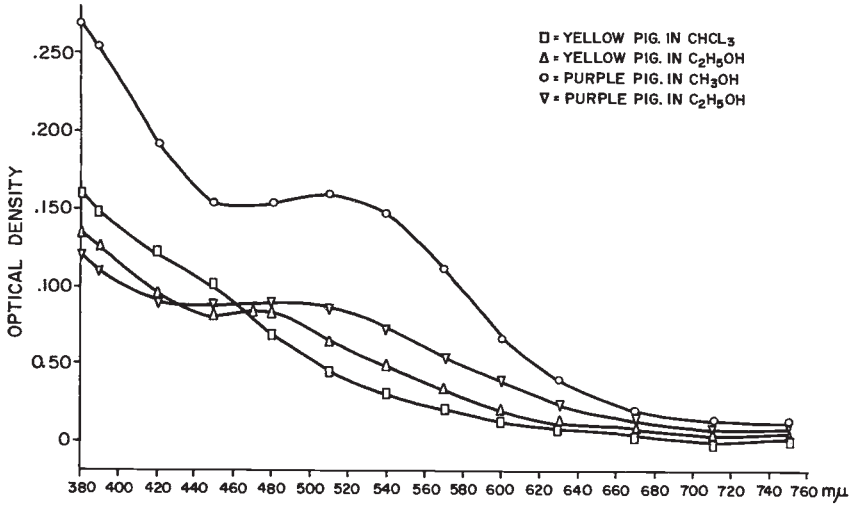

FIG. 6. Absorption spectra of the purple pigment of *M. cookei* in N/10 HCl, N/10 NaOH and pH 7.0 buffer in the visible region

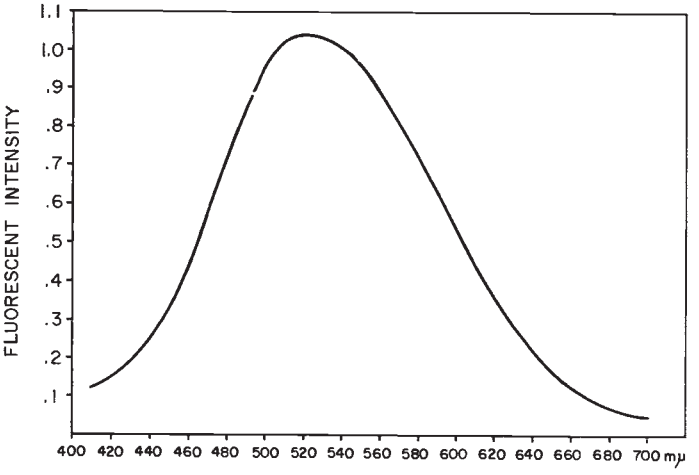

FIG. 7. Fluorescence spectrum of the bright yellow pigment of *M. cookei* in glacial acetic acid

mμ and at 300–310 mμ. In the visible, no absorption maximum is found in pH 7.0 buffer. There is a peak at 490–520 mμ in methanol, at 480 mμ in ethanol, at 400–410 mμ in N/10 HCl, and in N/10 NaOH there are two peaks, at 390 and at 470–510 mμ.

Upon dissolving the original yellow pigment in buffers of different pH, it was found that this pigment is yellow at pH 2–6.75, orange at pH 6.75–8.2, and purple at pH 8.2–10. The original purple pigment was rose at pH 2–5, violet at pH 5–9 and purple at pH 9–10.

Addition of sodium hydrosulfite to alkaline solutions of the pigments induced a color change from purple to a very faint yellow. The oxidized form could be restored by bubbling air through the solutions.

After obtaining a somewhat larger quantity of the crude chloroform extract of *M. cookei*, separation of the pigments by means of column chromatography was attempted using a variety of adsorbents. Sucrose proved to be highly satisfactory. The purple pigment was readily adsorbed at the top of the column, and the bright yellow fraction moved just above the solvent front. After the two pigments had separated, the column was extruded, the purple fraction was eluted with methanol, and the

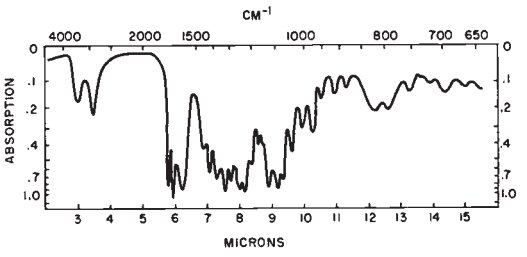

FIG. 8. Infrared absorption spectrum of the bright yellow pigment of *M. cookei*

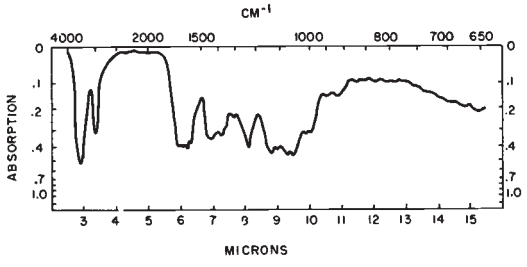

FIG. 9. Infrared absorption spectrum of the purple pigment of *M. cookei*

yellow fraction with chloroform. Both solutions were greatly concentrated under reduced pressure. Upon the addition of an excess of petroleum ether to the solution of the yellow pigment in chloroform, a considerable quantity of the yellow pigment was precipitated. This precipitate, orange in color, was collected by centrifugation.

The purple pigment was precipitated from methanol solution containing a small amount of chloroform by the addition of petroleum ether. The quantity of the yellow pigment was several times greater than that of the purple one.

Studies with a Fisher-Johns melting point apparatus indicated that the yellow pigment underwent thermal decomposition in the range 275–285°C., while the purple fraction did not decompose at 300°C., the maximum temperature possible with this apparatus.

The yellow pigment was subjected to the Hinsberg test for nitrogen (20) with negative results. The fluorescence spectrum of the yellow pigment in glacial acetic acid (Figure 7) shows a very broad fluorescence extending from 440 to 640 mμ, with a well defined peak at 520 mμ. The quantity of purple pigment available was unfortunately too small for these tests to be made.

Infrared absorption spectra of both the yellow pigment and the purple pigment, in KBr pellets, were taken with a Perkin-Elmer model 21 spectrophotometer, and are presented in Figures 8 and 9.

DISCUSSION

The presence in the infrared absorption spectra of both the yellow and purple pigments of *M. cookei* of a prominent peak at 3450 cm^{-1} indicates the presence of an —OH group. The peak in each spectrum at 2950 cm^{-1} indicates a $—CH_3$ group. A free —C=O group is shown by the peak at 1680 cm^{-1} and a bonded —C=O group by the peak at 1625 cm^{-1}. Peaks at 1460 cm^{-1} and 1380 cm^{-1} provide further evidence of the presence of a $—CH_3$ group. The peak at 1420 cm^{-1} is indicative of an —OH group, and those between 1215 and 1255 cm^{-1} would indicate that the —OH is attached to an aromatic ring structure. There is a general absence of strong absorption bands in the 750–650 cm^{-1} region.

The spectra of the yellow and purple pigments thus show considerable resemblance to each other. Further considerable points of similarity between these spectra and those of a number of anthraquinone derivatives may be found. The latter include 1,5 dihydroxy-3-methyl anthraquinone (Sadtler no. 3562), 1,6 dihydroxy-3-methyl anthraquinone (Sadtler no. 3563), 2,7 dihydroxy anthraquinone (Sadtler no. 3588), 1,7 dihydroxy-5-methyl anthraquinone (Sadtler no. 3593), 2-methyl anthraquinone (Sadtler no. 6342), 1,8 dihydroxy anthraquinone (Sadtler no. 14902), and 1,2 dihydroxy anthraquinone (Sadtler no. 15308). Inspection of these spectra (21) shows that the absence of a strong absorption band in the 750-650 cm^{-1} region indicates the presence of a substituent group in the beta position in each of the two lateral rings of the molecule (*i. e.*, at position 2 or 3 and at position 6 or 7). Consequently, both the yellow pigment and the purple pigment must bear at least one beta substituent in each of the two lateral rings. Further, it may be deduced that a beta —OH is required for the appearance of the prominent peak at 3450 cm^{-1}, since this peak is absent in compounds containing only an alpha —OH (in position 1, 4, 5, or 8). Consequently, both pigments must contain at least one —OH in a beta position. Further, the presence of a bonded —C=O group, as shown by the peak at 1625 cm^{-1} must indicate the presence of an —OH

group in an alpha position. The fluorescence of the yellow pigment indicates that it must have two alpha —OH groups in the 1,4 positions (22).

Several investigators, including Tate (3), Georg and Maechling (8), Mier (14) and McCabe and Mier (15), have suggested that the pigments of dermatophytes are polyhydroxy anthraquinones, but as Wirth *et al.* (13) have observed, there is very little evidence in direct support of this view. The present findings would appear to establish conclusively that the two pigments studied in detail, namely, the yellow and purple pigments of *M. cookei* are indeed polydydroxy-methyl-anthraquinones. In addition to the infrared absorption data, evidence for this conclusion includes the following:

(1) Like many polyhydroxy anthraquinones, these pigments have indicator properties and exhibit a redox phenomenon (4).

(2) The yellow pigment decomposes at a temperature which is within the melting point range of a number of anthraquinone derivatives (4).

(3) Both give the characteristic color reaction with magnesium acetate (19).

In their R_f values and ultraviolet absorption spectra, the two pigments studied agree well with the results of Mier (14) and McCabe and Mier (15).

Wirth *et al.* (13) reported the absence of nitrogen from the pigments which they obtained in crystalline form from *T. rubrum*, while nitrogen was present in both the yellow and purple pigments obtained from this organism by Zussman *et al.* (18). The absence of nitrogen from the yellow pigment of *M. cookei* as shown by the negative Hinsberg test represents a point of evidence against the melanoid nature of the pigments claimed by Zussman *et al.* (18) and is entirely consistent with the structure of a polyhydroxy-methyl-anthraquinone, as proposed by other workers.

SUMMARY

Microsporum cookei Ajello produces a purple pigment on Sabouraud's agar. By extraction with acetic acid, partition into chloroform and paper chromatography of the concentrated extract using chloroform:petroleum ether 7:3 as the developing solvent, it was shown that the pigment mixture contains a pale yellow fraction, a purple fraction and a bright yellow fraction. These components are identical with pigments present in *Trichophyton rubrum.*

Both the bright yellow pigment, which is present in greatest amount, and the purple pigment are pH indicators, and undergo reduction by sodium hydrosulfite and reoxidation by atmospheric oxygen. The two pigments may be separated in quantity by chromatography on a sucrose column, and have been obtained in solid form. Absorption spectra of both pigments in the visible and ultraviolet regions have been studied in a number of solvents.

The yellow pigment decomposes at 275–285°C., while the purple pigment failed to decompose at 300°C. The yellow pigment gives a negative reaction for nitrogen by the Hinsberg test. From evidence derived from a specific color reaction and infrared absorption spectra, it is concluded that both pigments are polyhydroxy-methyl-anthraquinones. Both pigments must have at least one beta substituent on each of the lateral rings, and must have at least one alpha hydroxyl and one beta hydroxyl. The yellow pigment is fluorescent and hence must have two alpha hydroxyls in the 1,4 positions.

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